

ON THE PROCESSES SHAPING SMALL-SCALE POPULATION STRUCTURE IN *RADIX BALTHICA* (LINNAEUS 1758)

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ABSTRACT

The evolutionary processes generating population genetic structures may differ depending on the spatial scale. We examined the population structure of the freshwater snail *Radix balthica* on a local scale in Saanen Valley, Switzerland. We used a combination of a direct approach by simulating dispersal with parameters gained out of experiments in an artificial pond and indirect molecular genetic approaches by using mitochondrial (COI) and nuclear (microsatellite) marker at all sites harbouring *R. balthica* in this restricted area.

Contrary to the expectations from the direct methods, populations in homogeneous water bodies were found to be genetically heterogeneous, independently of geographic distance between sites. Instead, several selfing lineages coexisted with little local gene flow among them.

The population structure observed in the valley could thus not be explained by active dispersal alone. It was rather characterized by independent, probably bird-mediated, colonisation events from several sources, in combination with high metapopulation dynamics and a mixed mating system preserving this structure. This population structure has rarely been reported before, but might nevertheless be typical for passively dispersed, patchily distributed taxa (e.g., freshwater invertebrates).

Key words: Basommatophora, computer simulation, neighbourhood, selfing, microsatellite marker, adaptation.

INTRODUCTION

Disentangling the processes governing spatial genetic structure is essential to understand the ecological and evolutionary dynamics of a species. Spatial genetic structure is shaped by the interaction of demography, natural selection, mating system and dispersal-mediated gene flow. However, the relative importance of these factors may vary according to spatial scale. On a local scale, active dispersal may play a more important role than long-range dispersal and vice versa on a broader scale.

Furthermore, a mixed mating system can substantially affect the distribution of genetic variation within and among populations (Charlesworth, 2003). Predominately selfing (sub-) populations are strongly influenced by drift due to a very low effective population size and are ultimately subject to genetic impoverishment (Jarne & Städler, 1995).

A recent study (Pfenninger et al., 2011) on the range-wide population structure of *Radix balthica* (Lymnaeidae) revealed a population structure characterised by the absence of isolation-by-distance, together with rather isolated and genetically depauperate populations compared to the variation present in the entire range. This seemingly paradoxical pattern was explained by distance-independent, passive long-range colonisation and high population turn-over rates, low gene-flow and/or strong drift, enhanced by the mixed mating system of the species (Pfenninger et al., 2011). This pattern of population structure, probably typical for passively dispersed, patchily distributed taxa (e.g., freshwater invertebrates), is likely to impede local adaptation (Kawecki & Ebert, 2004).

Radix balthica occurs in permanent slow-flowing rivers and stagnant water bodies, without requiring a particular substratum or high water quality (Økland, 1990; Glöer & Meier-Brook,

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1998). The individual active dispersing capacity is restricted to the water body the snail lives in. The egg clutches are attached to structures, and there is no free larval stage allowing dispersal (Glöer & Meier-Brook, 1998). Still, *R. balthica* is widely distributed over northwestern Europe (Pfenninger et al., 2006), implying extensive passive transport, as suggested for many freshwater taxa (e.g., Frisch et al., 2007). Many gastropods have a mixed mating system with outcrossing and selfing both common (Jarne & Städler, 1995; Meunier et al., 2001), which is also true for *R. balthica* (Pfenninger et al., 2011).

To understand the regional and local population structure of *R. balthica* in relation to the range-wide structure, we used direct (dispersal studies) and indirect (molecular genetic) methods at all sites harbouring *R. balthica* in a restricted area. In particular, we asked the following questions:

What are the expectations for local (intra water body) population structure of *R. balthica* based on active dispersal, and how do they correspond to the structure as inferred from molecular markers?

Are the populations connected by gene-flow on a regional scale, that is, in an area of 56 km²?

How does the inferred structure relate to the large-scale population over the whole area of distribution structure?

METHODS

Direct Estimation of Population Structure

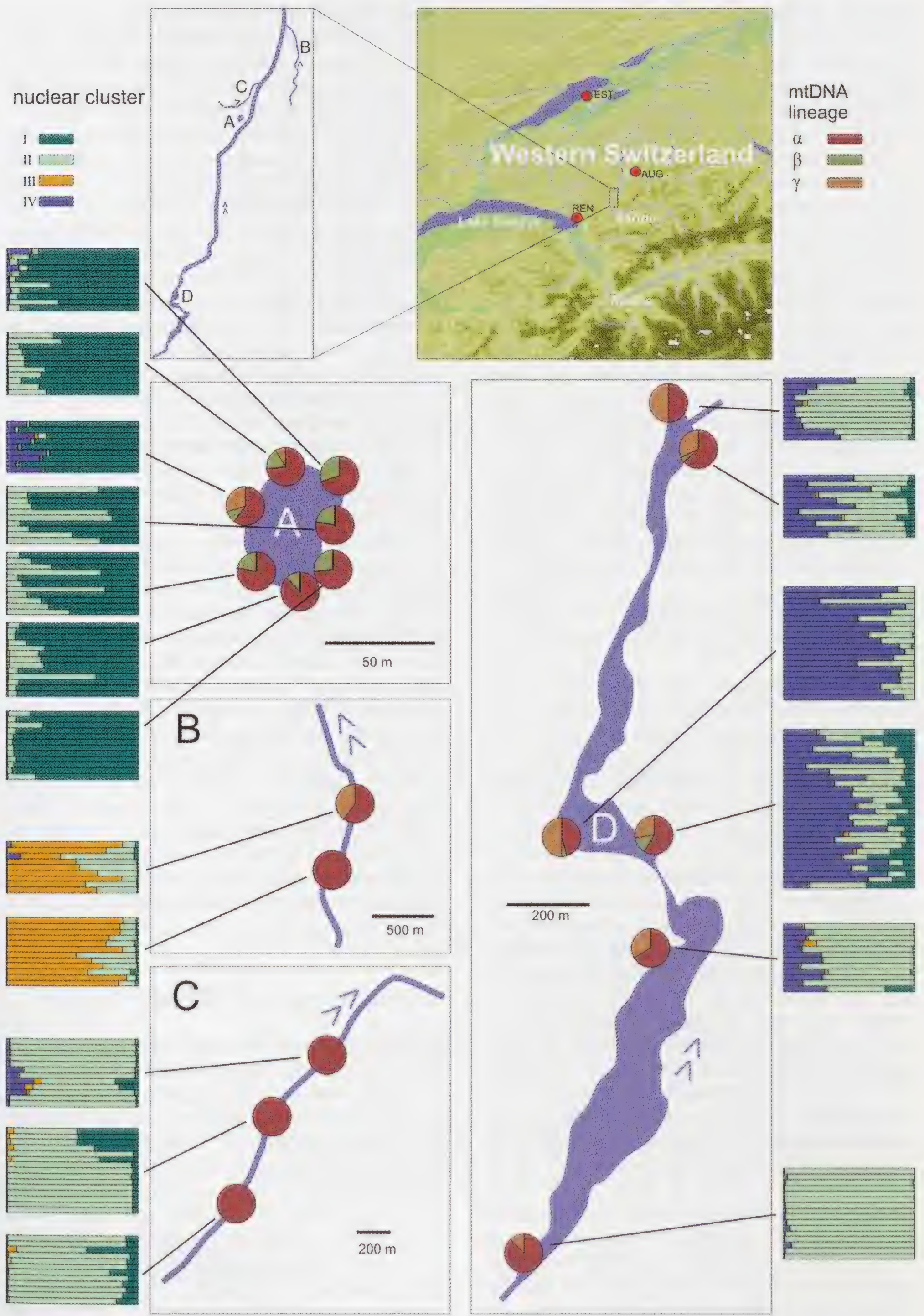
We conducted a series of studies that parameterised computer simulations to estimate the life time dispersal based on daily movements. This required knowledge of (i) the frequency distribution of daily movement distances, (ii) position preference in the habitat, (iii) dependence of movement directions on previous movement and (iv) dependence of positions between individuals (Pielou, 1969). Additionally, the seasonal water temperature is an important factor for the activity of freshwater snails and was taken into account.

Pond Set-up and Recording of Daily Net Movement Distances: To obtain a distribution of daily movement distances, we set up an artificial pond of 4 x 4 m in size. The study was conducted in June 2010, during which the mean daily maximum temperature was 18.8°C. We used tap water to fill the pond initially to 20 cm depth and inoculated it with 50 litres of natural pond water. As substratum, we inserted a 2 cm layer of sieved and dried local loam. We let the pond settle for two weeks, during which it was naturally filled up by rainwater to a depth of 30–35 cm and accumulated sufficient debris as a food source for the snails. Thirty individually marked adult snails > 10 mm from a laboratory stock population were released in the middle of the pond. After an acclimatisation period of one week, we recorded the position of living individuals every 24 hours relative to one corner of the pond for a period of 21 days. The daily net dispersal distances between two records were calculated as straight lines. We also recorded the dispersal directions and their changes between successive days. Individuals not surviving the measurement period were discarded from the analysis. The distribution of daily net movements over the study period was fitted to an exponential function.

Testing for a Preferred Position in the Habitat: Observations in the field and during the study suggested that the snails tended to stay close to the rim. We therefore measured the distance of each snail to the closest shoreline on the last day of the survey, to compare the observed distribution with that expected under a computer simulated movement model. For this simulation, a virtual snail was set at a random position within a virtual 4 x 4 m pond. A random variate was drawn from the observed daily net movement distribution and assigned to a random direction. If a snail would have crossed the rim of the pond with a movement in the simulation, it was reflected at the rim with the opposite direction and moved on the remaining distance. This was repeated 21 times for each snail and after the last move, the distance to the closest rim was recorded. The simulation was run 1,000 times for 29 snails.

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FIG. 1. Spatial distribution of the sampling sites along the Saane valley in Switzerland (square) and three known neighbouring populations (red dots). The position of the 18 sampling sites within the water bodies (labelled A–D) are indicated by coloured circles representing the fraction of the mtDNA lineages (α , β , γ), together with the cyto-nuclear composition of the individuals sampled. Vertical bar represents a single individual. Column shows the estimated nuclear composition of the multilocus microsatellite genotype (dark green = cluster I, light green = cluster II, yellow = cluster III, blue = cluster IV) as indicated by STRUCTURE.



Test for Independence of Previous Moves and of Individual Positions: Independence of movement directions from previous moves was tested by comparing the number of changes in consecutive movement directions to random expectations with a χ^2 test.

To test whether the snail's positions (and thus the movements) were independent from each other or whether they tended to aggregate or avoid each other, we recorded the average distance between the snails after 21 days and compared it to the expected distribution under a movement simulation model. We used basically the same simulation model as described above with two modifications. First, as previous results indicated that the snails moved almost exclusively along the rim of the pond, we considered only the distance vectors parallel to the rim from the observed daily net movement distribution. Second, because consecutive moves were not independent from previous moves, direction changes were randomly assigned according to the observed direction-change probability distribution. The average distance along the rim between all snails was recorded after 21 days. The simulation was run 10,000 times for 29 snails.

Temperature Dependence of Activity: To infer the temperature dependence of activity in *Radix*, individually marked snails ($n = 10$) of each of two laboratory stock populations were put in a 30 litre glass aquarium filled with aerated water in a climate chamber (one aquarium for each stock population). Food ad libitum (powdered TetraMin fish-food, Tetra) was provided. We increased the ambient temperature of the chamber from 2°C to 34°C in steps of 2°C/h. When the next temperature level was reached, which took about one hour, the temperature was held constant for the measurement of individual movements for one hour. Total distances covered in this hour by each individual were recorded. Movement per hour was averaged over both investigations. To establish the relationship between activity and water temperature, we calculated a linear regression between movement (dependent variable) and temperature (independent variable).

Modelling Life-Time Dispersal: The results of the simulations described above were used to design and parameterise a life-time dispersal model for *Radix*. It was assumed that the snails have a life span of approximately 260 days. This matches field observations of an

annual life cycle (Dillon, 2000) and equals the number of the days in the River Saanen Valley with a mean temperature above 5°C, excluding the time for hibernation. To simulate the life time dispersal distance of a snail in a two dimensional habitat of unrestricted size, it was initially placed at position zero. A random variate from the exponential function of the observed daily movement distance distribution along the shore line was drawn. To take the temperature dependence of movement activity in *Radix* into account, the assumed life time was divided into eight periods of 30 days (corresponding to the months March to November) and two shorter periods of ten days at the beginning and the end (each during February and December). The determined temperature-dependent activity function was then used to determine the correct drawn movement distance according to the average monthly mean temperature in the Saanen Valley. Climate data for the Saanen Valley were taken from <http://www.meteoschweiz.admin.ch>. Whether the direction of movement for the subsequent day changed or not was randomly determined according to the observed direction change probability. This procedure was repeated 260 times and the distance of the final position to the starting point of the snail recorded. We simulated 100,000 snails in total.

Wright (1946) calculated the neighbourhood size for a linear-distributed population in a two-dimensional habitat as $N = \sqrt{2} p \cdot \sigma \cdot d$, with σ being the variance of dispersal along the habitat from parents relative to their offspring at the same developmental stage and d as the observed density of breeding individuals. The strip length where parents potentially can originate from and no population differentiation can be expected thus equals about 3.55σ (Wright, 1946).

Indirect Estimation of Population Structure

Sampling Design: Sampling was conducted in a part of the river Saanen Valley, Switzerland. We searched for *Radix* populations in all natural and artificial water bodies in the valley between Montbovon (46.628°N, 7.044°E) and the Lac de la Gruyère (46.623°N, 7.101°E) and below 1,000 m altitude. Two hundred and seventy individuals from 18 sites were sampled. *Radix* were found in only four out of 35 water bodies: two small creeks, a gravel pit and a reservoir of the Saanen river (Table 1, Fig. 1). Along the creeks where *Radix* occurred, all adult snails (> 8 mm) that could be found were sampled

TABLE 1. Overview of the 18 sampled localities, the *STRUCTURE* clustering (A, B, C and D) with the exact coordinates (°N and °E), the age of the waterbody and the number of individuals sampled (N_t). Mitochondrial lineage present at each locality (α , β , γ). Genetic diversity was estimated by mitochondrial DNA marker COI for n individuals (M_{indmt}) with haplotype diversity (D_{hap}), mean Nei's gene diversity (D_{mt}) with standard deviation (s.d.) and by nuclear DNA markers (Sallingør & Pfenninger, 2009) for n individuals (N_{indnc}). (N_a = number of alleles; D_g = mean Nei's gene diversity; s.d. = standard deviation; H_o = heterozygosity observed; H_e = heterozygosity expected. The inbreeding coefficient (F_{IS}) was calculated for each sampling site and the selfing rate s were estimated by three methods: F_{IS} , g_2 and ML.

Locality	waterbo- dy type	°N	°E	age (years)	lineage	D_{hap}	D_{mt}	s.d.	N_a	N_{indmc}	s.d.	D_g	s.d.	H_o	H_e	F_{Is}	$s(F_{Is})$	$s(g_2)$	$s(M_L)$
A	gravel-pit	46.54937	7.07865	~ 10	α, β	0.004	0.002	11 2.25	1.21	0.348	0.258	0.159	0.332	0.521	0.362	0.000	0.000	0.018	0,018
A1		46.54943	7.07869	~ 10	α, β	0.200	0.002	11 2.25	1.21	0.348	0.258	0.159	0.332	0.521	0.362	0.000	0.000	0.018	0,018
A2		46.54939	7.07872	~ 10	α, β	0.222	0.002	10 2.00	0.82	0.300	0.230	0.106	0.284	0.627	0.521	0.901	0.901	0,837	0,837
A3		46.54933	7.07872	~ 10	α, β	0.200	0.002	15 2.50	1.50	0.341	0.251	0.114	0.328	0.652	0.538	0.000	0.000	0,051	0,051
A4		46.54939	7.07872	~ 10	α, β	0.375	0.001	10 2.25	1.21	0.294	0.243	0.150	0.279	0.462	0.312	0.231	0.226	0,226	0,226
A5		46.54931	7.07860	~ 10	α, β	0.200	0.001	11 2.38	1.13	0.379	0.209	0.155	0.361	0.571	0.416	0.535	0,506	0,506	0,506
A6		46.54938	7.07852	~ 10	α, β, γ	0.250	0.002	9 1.88	1.17	0.381	0.272	0.215	0.360	0.403	0.188	0.320	0,416	0,416	0,416
A7		46.54943	7.07857	~ 10	α, β, γ	0.182	0.001	11 2.13	0.41	0.314	0.250	0.046	0.300	0.847	0.801	0.764	0,756	0,756	0,756
B	creek	46.55088	7.09522 > 10,000		α	0.154	0.000	14 2.00	0.49	0.363	0.181	0.205	0.350	0.414	0.209	0.359	0,140	0,140	0,140
B1		46.55963	7.09479 > 10,000		α	0.154	0.000	14 2.00	0.49	0.363	0.181	0.205	0.350	0.414	0.209	0.359	0,140	0,140	0,140
B2		46.56065	7.09578 > 10,000		α, γ	0.600	0.002	7 1.63	0.45	0.239	0.210	0.110	0.221	0.502	0.392	0.764	0,576	0,576	0,576
C	creek	46.55066	7.07707 > 10,000		α	0.125	0.001	13 2.13	1.50	0.251	0.304	0.087	0.241	0.639	0.552	0.764	0,633	0,633	0,633
C1		46.55093	7.07726 > 10,000		α	0.125	0.001	13 2.13	1.50	0.251	0.304	0.087	0.241	0.639	0.552	0.764	0,633	0,633	0,633
C3		46.55196	7.07832 > 10,000		α	0.200	0.000	14 1.85	0.50	0.205	0.200	0.135	0.197	0.315	0.180	0.645	0,545	0,545	0,545
C5		46.55326	7.07938 > 10,000		α	0.083	0.000	12 3.38	0.50	0.235	0.275	0.099	0.225	0.560	0.461	0.442	0,568	0,568	0,568
D	reservoir	46.49670	7.05196	~ 100				98											
D1		46.49459	7.05167	~ 100	α, γ	0.500	0.002	11 4.38	2.00	0.443	0.200	0.288	0.420	0.314	0.026	0.000	0,019	0,019	0,019
D2		46.50536	7.05272	~ 100	α, β, γ	0.304	0.002	13 4.38	2.60	0.560	0.165	0.317	0.539	0.412	0.095	0.071	0,071	0,071	0,071
D3		46.50675	7.05248	~ 100	α, γ	0.800	0.004	10 3.50	1.92	0.500	0.124	0.306	0.474	0.354	0.048	0.419	0,379	0,379	0,379
D4		46.48808	7.04774	~ 100	α, γ	0.375	0.001	16 2.88	1.13	0.445	0.223	0.263	0.430	0.388	0.125	0.209	0,319	0,319	0,319
D5		46.49727	7.04901	~ 100	α, β, γ	0.363	0.002	20 4.50	2.00	0.541	0.179	0.313	0.527	0.406	0.093	0.291	0,199	0,199	0,199
D6		46.49707	7.05188	~ 100	α, β, γ	0.270	0.002	28 5.13	2.85	0.586	0.187	0.359	0.576	0.377	0.177	0.143	0,052	0,052	0,052
all								0.005	0.001	235 4.39	2.36	0.389	0.120	0.214	0.486	0.510	0.346	0.332	0,153

along a stretch of 5 m, every 50 m. In the gravel pit, all adult snails were sampled and pooled per 10 m shoreline. If the density was high, a maximum of 22 adult snails were taken per section. At the reservoir, all accessible parts of the shoreline were searched and 11–30 snails were sampled at places where *Radix* were present. Individuals were immediately preserved in 100% ethanol. The distances between sampling sites were measured along the shoreline (Table 2) for comparison to the neighbourhood size calculated from the simulation of lifetime dispersal.

Microsatellite and Mitochondrial Haplotype Analysis: DNA was extracted using a glass fibre DNA extraction method following the protocol of Ivanova et al. (2006). About 1 mm³ of alcohol-preserved snail tissue was mixed with vertebrate lysis buffer containing 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.5% SDS and 20 mg per ml proteinase K. Samples were incubated over night at 56°C. After digestion, samples were mixed with binding buffer (BB), composed of 6 M guanidinium thiocyanate, 20 mM EDTA, 10 mM Tris-HCl (pH 6.4), 4% Triton X-100, transferred into a Pall1 glass fibre plate (GF plate, AcroPrep™), placed on top of a 2 ml square well block and centrifuged to bind DNA to the GF membrane. DNA washing was carried out in two steps: (1) Protein wash buffer: BB and EtOH 96%, (2) Wash buffer: 60% EtOH, 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 0.5 mM EDTA (pH 8.0). After washing, residual ethanol was evaporated by incubating the GF plate at 56°C for about 30 minutes. DNA was eluted by incubating each GF membrane with 50 µl of prewarmed (56°C) ddH₂O followed by centrifugation using a 96-well collection plate.

Cytochrome oxidase subunit I (COI) fragments were amplified using polymerase chain reaction (PCR) performed with Invitrogen Taq DNA polymerase and universal primers (Folmer et al., 1994). Sequencing reactions were performed using the ABI Prism Big Dye terminator kit (Perkin-Elmer, Norwalk, CA, USA). Sequenced fragments were read on an ABI Prism 3730 automated capillary sequencer (Applied Biosystems).

All snails were genotyped for eight highly polymorphic microsatellite loci (Salinger & Pfenninger, 2009). Multiplex Microsatellite amplification was carried out using the Quiagen Type-it™ microsatellite PCR Kit with fluorescent dye labeled forward primers (Salinger & Pfenninger, 2009). PCR products were

separated and visualized on an ABI Prism 3730 sequencer (Applied Biosystems) with GeneScan™ 500-LIZ™ size standard (Applied Biosystems). Microsatellite allele lengths were recorded using GENEMAPPER 4.0 software (Applied Biosystems).

Genetic Variability: Mitochondrial sequences of the COI-fragment of 482 bp length were aligned using ClustalW (Thompson et al., 1994) as implemented in MEGA 4.0 (Tamura et al., 2007) and corrected by eye. We used this alignment to calculate a parsimony haplotype network in TCS 1.21 (Clement et al., 2000). We nested haplotypes in a hierarchical fashion as described by Pfenninger & Posada (2002). Identification of the sampled snails as *R. balthica* was done by comparing the respective barcodes to the reference sequences of Pfenninger et al. (2006; GenBank Accession no. DQ980030–DQ980193) using a neighbour-joining tree and monophyly with the previously identified haplotypes. This was necessary, because other *Radix* species are present in the region and specific identification is only possible with molecular markers (Pfenninger et al., 2006). Mitochondrial genetic distances among the water bodies were calculated as Nei's standard genetic distances (D_{mt} ; Nei, 1972) with MEGA 4.0 (Tamura et al., 2007).

We used GenePop (Raymond & Rousset, 1995a) to determine allele and genotype frequencies of the microsatellite markers, for the different sampling sites, the four water bodies and the entire sample. To investigate population differentiation, F_{ST} was calculated using *FSTAT* 2.9.3.2 (Goudet, 1995). *FSTAT* was also used to estimate the expected (H_E) and observed heterozygosities (H_O) for each sampling site and to test for deviation from Hardy-Weinberg Equilibrium among sampling sites within water bodies (1,000 permutations, two-sided test). Bonferroni correction for multiple tests was applied to each of these tests. Nuclear genetic distances among the water bodies (D_G ; Nei, 1972) were calculated using Genetix 4.04 (Belkhir et al., 1996–2004). The microsatellite data were also subjected to a factorial correspondence analysis with Genetix 4.04 (Belkhir et al., 1996–2004) to visually explore the distribution of genetic variation.

Sensitivity Test: Because our data set contained sampling sites with relatively few individuals, we conducted two initial simulation studies to assure that observed deviations from the null hypothesis of no population structure are

not due to sampling artefacts. First, we simulated 300 times a truly panmictic population of 200,000 individuals with a diploid genotype of eight independent loci with the same possible number of allelic states as observed in our sample of natural populations, respectively, using EASYPOP (Balloux, 2001). The mutation rate was set to 0.05 and a model with mutations equally likely to occur at any of K possible sites (KAM) was chosen. After 1,000 generations, a dataset from the final generation of 235 individuals was generated, divided into 18 groups of the same size as our sample of natural populations. A second dataset was generated almost identically according to the sampling parameters at sampling site A. The number of individuals was set to 77 and the number of groups was set to 7.

The resulting datasets were tested for deviation from the null hypothesis of no population structure with an AMOVA (Analysis of Molecular Variance, 10,000 replicates) in Arlequin 3.11 (Excoffier et al., 2005).

Inference of Population Structure: Assignment of individuals to genotype clusters without a priori classification was performed with STRUCTURE (Pritchard et al., 2000). All MCMC runs were repeated five times for each value of K ($K = 1$ to $K = 20$) for 1,000,000 generations with 50,000 burn-in steps, applying the admixture model. We also used a new model developed for STRUCTURE that integrates information about sampling locations. This can be useful if the cluster identification without a priori information would not suffice to detect clusters in a dataset (Hubisz et al., 2009). We used the same settings as in the model without the location-prior. Both models were analysed using the maximum $\text{LnP}(D)$ value. $\text{LnP}(D)$ is the log likelihood of the observed genotype distribution in K clusters.

F_{ST} among water bodies was calculated with Genetix 4.04 (Belkhir et al., 1996–2004). An exact test of population differentiation (Raymond & Rousset, 1995b) was performed using Arlequin 3.11 between all pairs of sampling sites within the respective water bodies, with 1,000,000 steps of Markov Chain length and 1,000,000, dememorization steps.

Isolation by Distance: We tested for Isolation-by-Distance among sampling sites using a Mantel's test (Mantel, 1967). A correlation between $F_{ST} / (1 - F_{ST})$ and the natural logarithm of pair-wise geographical distances was obtained using the ISOLDE

program of the GENEPOP package (Raymond & Rousset, 1995a; Rousset, 2008).

Estimation of Selfing Rates: Genotype frequencies at codominant marker loci may convey information on mating systems (David et al., 2007). The classical approach is to measure heterozygote deficiencies and obtain the selfing rate s from $F_{IS} = s / (2-s)$, assuming inbreeding equilibrium. A drawback of this estimate is that heterozygote deficiency may not only be due to selfing. Biparental inbreeding and partial dominance as well as such technical artefacts as null alleles also increase the proportion of observed homozygotes. Therefore, in addition to this classical approach, we used a method implemented in RMES (David et al., 2007) to derive estimates of $s(g_2)$, based on a point estimate of the second-order heterozygosity disequilibrium and a maximum likelihood of the whole distribution based approach of $s_{(ML)}$. These estimates are independent of F_{IS} and less influenced by technical biases.

Assignment Tests: To compute the probability of each individual being derived from external populations or being a resident (i.e., not a first-generation migrant) in the Saanen Valley, we used GENECLASS2 (Piry et al., 2004). As a reference dataset we used 80 populations, covering the approximate species range, from a previous study on *R. balthica* (Pfenninger et al., 2011). This software computes genetic assignment statistics to assign or exclude reference populations as the origin of individuals on the basis of multilocus genotype data. It is thus also possible to obtain direct estimates of dispersal through the detection of immigrant individuals (Rannala & Mountain, 1997; Paetkau et al., 2004). For the assignment test and the test for first generation migrants we selected the likelihood ratio $L_{\text{home}} / L_{\text{max}}$ and a Bayesian approach by Baudouin & Lebrun (2001) with an assignment threshold of scores of 0.01 and tested for probabilities using a simulation algorithm by Paetkau et al. (2004) with 100,000 simulated individuals and an alpha error set to 0.05. This approach provides a probability estimate of exclusion from or inclusion in a population, given only the genotype of the animal in question, and estimates of the allele frequency of the population in question. This simulation approach is sensitive to sample size and the degree of genetic differentiation among the populations. The significance of results was estimated with Monte Carlo re-sampling techniques as implemented in GENECLASS2 (Paetkau et al., 2004).

RESULTS

Movement Dispersal Patterns

During the 21 days of the study, 402 moves were recorded from 27 snails. Daily distances ranged from 0.01 to 3.86 m, with a median of 0.43 m. Distances covered in 21 d ranged from 3.22 m to 23.02 m, with a mean of 13.60 ± 4.96 m (mean $\pm$ s.d.). The frequency distribution of daily dispersal distances fitted a function with exponential decay ($y = 85.7 e^{-0.23x}$; $r^2 = 0.88$, $p < 0.001$, $n = 1,000$).

The observed average distance between individuals after 21 days was 5.25 ± 3.88 m (mean $\pm$ s.d.). Average inter-individual distance from a random movement simulation was 5.53 ± 3.95 m (mean $\pm$ s.d.). The observed value falls well within the 95% confidence interval. The null hypothesis of random distribution could thus not be rejected.

Movement along the rim was preferred over movement away from the rim ($\chi^2 = 47.12$; $n = 395$; $d.f. = 3$; $p < 0.001$; comparison with a uniform distribution). The snails lived and moved almost exclusively close to the rim of the artificial pond. Their observed distribution relative to the rim was significantly different from the expected distribution obtained from

a random movement simulation. Directions of second moves were dependent on direction of the first moves ($\chi^2 = 22.69$; $n = 365$; $d.f. = 2$; $p < 0.001$; comparison of deviation from first move with a uniform distribution). Snails maintained directions on consecutive days in 62% of cases. Random movement could thus with respect to direction not be assumed.

Movement of *R. balthica* was strongly temperature dependent. A linear regression for the distance moved per hour against water temperatures was highly significant (Fig. 2).

Computer simulated mean effective lifetime dispersal in a two-dimensional habitat was 48.25 m (s.d. = 35.75 m) in 260 d. Minimum simulated effective lifetime dispersal distance was 0.11 m and maximum distance was 229.62 m.

Direct Estimation of Population Structure

The standard deviation of life time dispersal was 35.75 m. The spatial extent of a neighbourhood was thus 126.9 m of shore line. If we compare this to the distances between the sampling locations (Table 2), we do not expect population differentiation at least among sampling points in water body A and between sampling points D2 and D3.

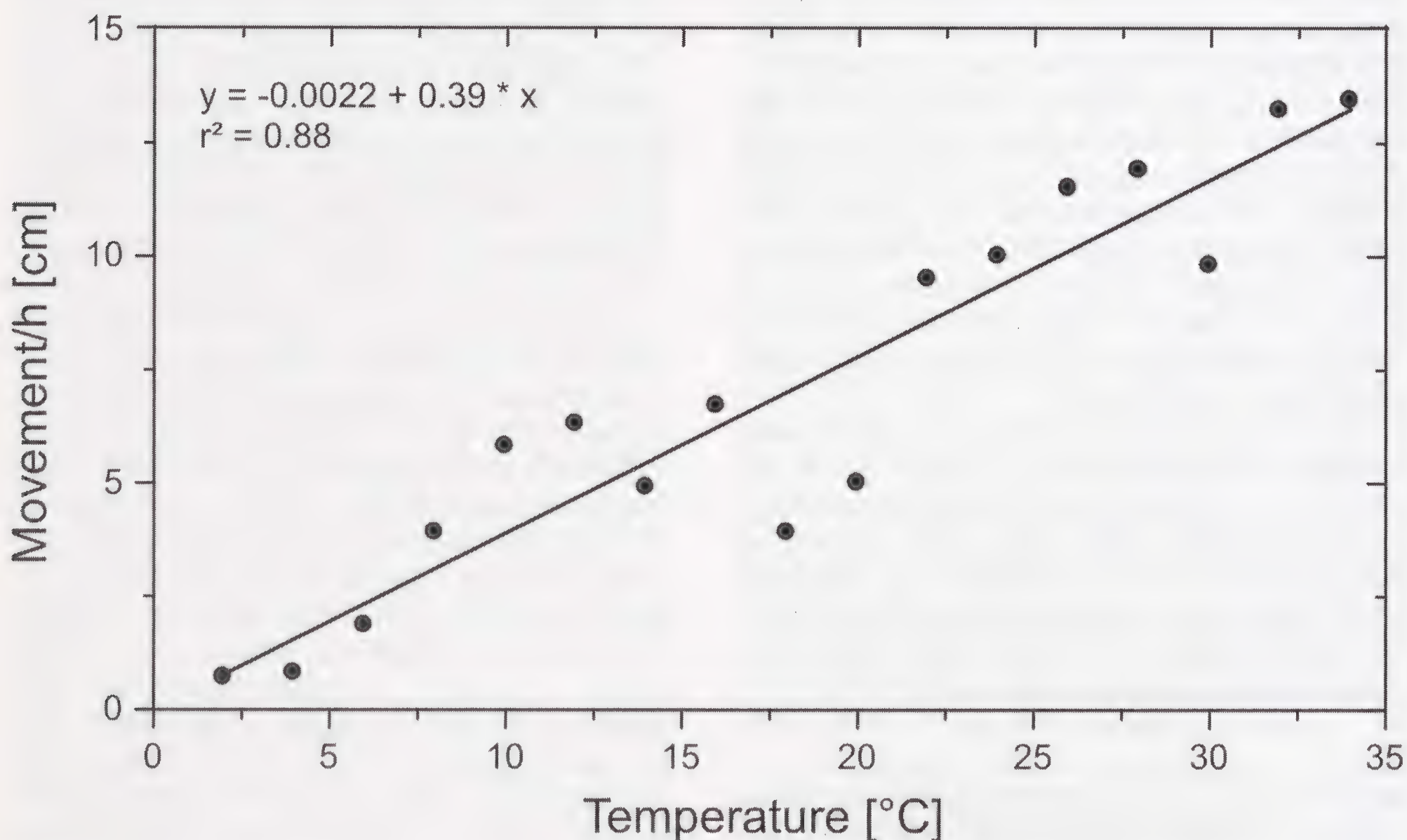


FIG. 2. Linear regression of *R. balthica* movement per hour against water temperature. Movement per hour are averages measured from 10 snails. The regression was highly significant ($p < 0.0001$).

TABLE 2. Distances in meters between the sampling points along the shoreline within the water bodies. Asterisks show significance level of an exact test of population differentiation (*p < 0.05; **p < 0.01; ***p < 0.001) between all pairs of samples within the water bodies.

	A1	A2	A3	A4	A5	A6	A7	B1	B2	C1	C2	C3	D1	D2	D3	D4	D5	D6
A1		25	39*	53	75	48	24*											
A2	25		14	28	50	76	48**											
A3	39*	14		14	36	67	63**											
A4	53	28	14		22	53	76*											
A5	75	50	36	22		31	56*											
A6	48	76	67	53	31		25*											
A7	24*	48**	63**	76*	56*	25*												
B1									418									
B2								418										
C1											342	587						
C2										342		248						
C3										587	248							
D1														1748	1621	939*	482**	2256**
D2													1748		125	2607*	1286***	1250**
D3													1621	125		2567*	1161*	1364*
D4													939*	2607*	2567*		1390*	1404*
D5													482**	1286***	1161*	1390*		2467
D6													2256**	1250**	1364*	1404*	2467	

Incidence of *Radix balthica* in the Sampling Region

Despite the presence of many seemingly suitable water bodies in the Saanen Valley, *Radix* snails were found only in a small fraction of them (4 out of 35). These tended to be the larger ones, although the species was absent from one of the larger gravel pits in the immediate vicinity of water body A.

Mitochondrial Haplotype Variability

At least 482 bp of the COI-gene fragment were obtained and manually aligned for 236 individuals from the Saanen Valley. All were identified as *R. balthica* by comparing the sequences to the reference sequences (data not shown). There were 28 polymorphic sites in our sample and 19 haplotypes (GenBank, accession no. GU735965-GU736200). Haplotypes were designated according to the nomenclature of Cordellier & Pfenninger (2009). Statistical parsimony analyses clustered these haplotypes into three lineages (α , β , γ , Fig. 3). Lineage α included 9 haplotypes from more than half the individuals analyzed ($n = 169$), β comprised 4 haplotypes from 23 individuals and γ included 6 haplotypes from 34 individuals. The distribution

of haplotype lineages among sampling sites is shown in Figure 1. Genetic diversity within lineages was between 0.4 and 5.9% and genetic divergence among lineages ranged from 0.1 to 7.1%. The genetic diversity at each sampling site and within each water body was measured by two estimators, haplotype diversity (D_{hap}), which ranged from 0.08 to 0.8, and genetic diversity (D_{mt}), which ranged from 0 to 14% between sampling sites with a mean of 5% differentiation (Table 1).

Nuclear Microsatellite Variability

Two-hundred-and-thirty-five individuals were successfully genotyped at 8 microsatellite loci. The number of alleles per locus ranged from 4 to 16, with an average of 4.4 (Table 1). The mean number of alleles (N_a) ranged from 1.85–5.13; Nei's mean genetic diversity had values from 0.2 to 0.59 with an overall mean of 0.39. All microsatellite loci showed consistent heterozygote deficiency with an overall observed heterozygosity of 21.4% and an expected heterozygosity of 48.6% for the sampling sites. Values of expected heterozygosity (H_e) ranged from 0.19–0.58 and observed heterozygosity (H_o) from 0.1–0.36. Detailed values are shown in Table 1. Significant devia-

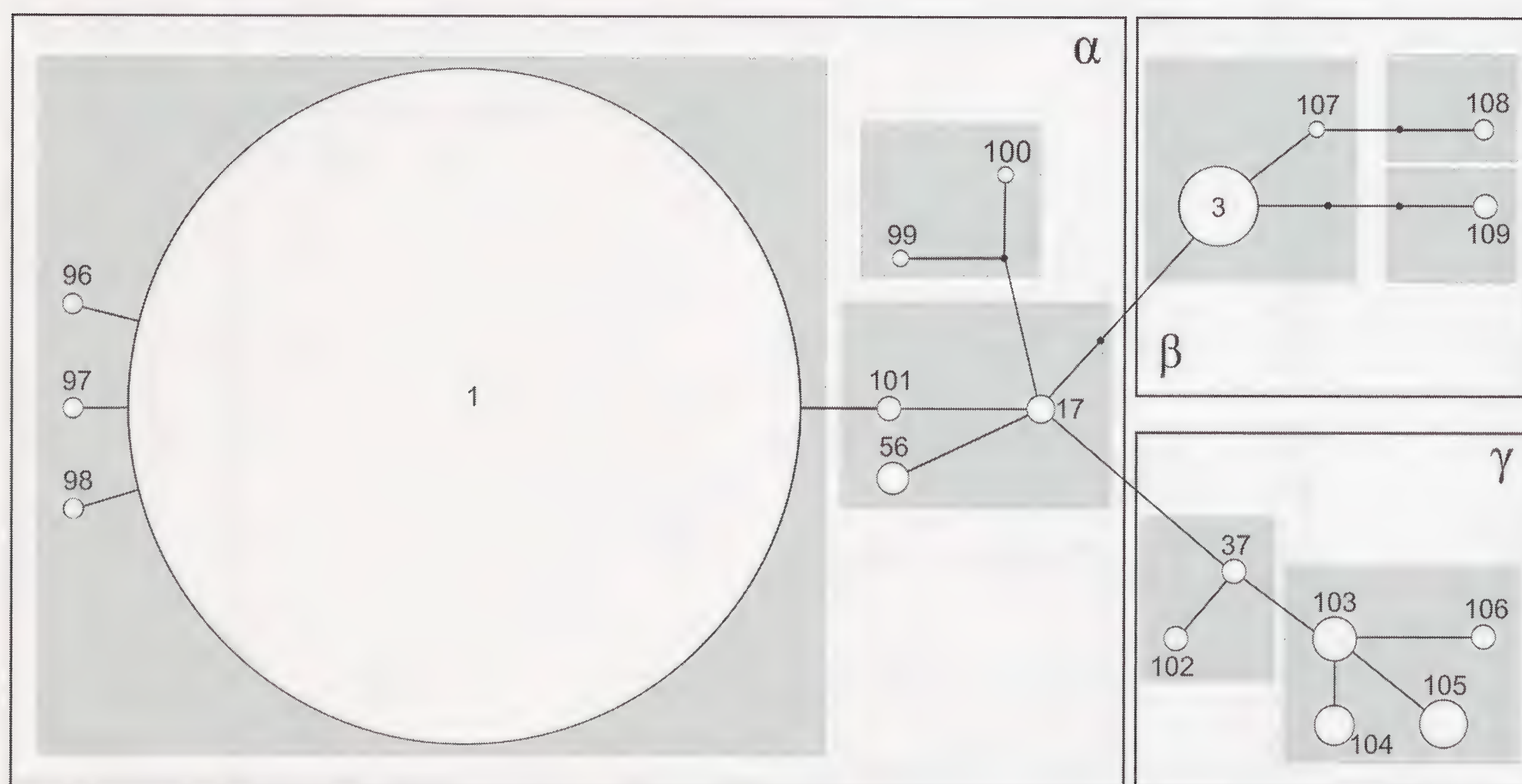


FIG. 3. Minimum spanning network of *R. balthica*. Each circle represents a haplotype, its size being proportional to the frequency of a certain haplotype. Connecting lines represent one mutational step. Small black circles depict haplotypes that were not sampled. The lineages are named α , β , γ .

TABLE 3. The grouping of the nuclear genotypes into four clusters (I, II, III and IV) is based on the *K* from the *STRUCTURE* analysis. Lineage α , β and γ refer to the inferred mitochondrial lineage. The *RXC* exact contingency table test on independence was significant (*d.f.* = 6, χ^2 = 16.6, *p* < 0.001).

	α	β	γ
I	57	8	5
II	41	6	4
III	8	6	8
IV	39	1	13

tions from Hardy-Weinberg equilibrium were observed in nearly half the loci in the sampling points, even after Bonferroni’s correction. We attribute this systematic deviation to a mixed mating system common in pulmonates (Jarne & Stadler, 1995) and described for *R. balthica* (Salinger & Pfenninger, 2009).

Cyto-Nuclear Association Test

There was a weak, albeit significant association of the two nuclear clusters (inferred from the ΔK) with mitochondrial haplotype lineages (*N* = 196, *p* < 0.001, χ^2 = 16.6, *d.f.* = 6, exact 4x3 *RXC* contingency test). Slightly less individuals of nuclear cluster I contained lineage γ haplotypes than expected by chance. The same was true for nuclear cluster III individuals with lineage α haplotypes (Table 3).

Sensitivity Test

The simulated datasets indicated that the actual number of loci used, their variability and the applied sampling design were capable of correctly keeping the null hypothesis of no

population differentiation if it is true. This was true in 291 out of 300 tested cases, for the full dataset. For the reduced dataset, equivalent to water body A, this was true in 287 out of 300 tested cases, corresponding in both cases to a type I error of less than 0.05.

Inference of Population Structure

Applying a location prior to the *STRUCTURE* analysis found the highest mean likelihood value for *K* = 4. Seventy individuals were attributed to the first cluster (I), 51 to the second (II), 22 to the third (III), 53 to the fourth (IV). However, even though the number of clusters found corresponded to the number of water bodies examined, there was no clear association between them (Fig. 1).

The overall *F*_{ST} among all sampling sites was 0.15, ranging from 0.08 to 0.25. Results of the test for exact population differentiation within the water bodies showed highly significant differentiation between several sampling points (Table 2).

We found no pattern of overall isolation-by-distance (*r*² = 0.0005, Mantel test *p* = 0.491).

Population Assignment Test

Applying the simulation approach, 213 out of 235 animals (91%) were genetically assigned as residents rather than immigrants. Assignment was probable to three outside populations for 13 individuals to a neighbouring population in northern Switzerland (AUG – 46.615°N, 7.181°E) and seven and two individuals to either of two populations in southern Sweden (SEM – 57.155°N, 16.419°E; EDA – 59.834°N, 17.878°E).

Testing for first generation migrants highlighted a single individual with most likely external origin in AUG. Eight individuals identified as first

TABLE 4. An analysis of molecular variance (AMOVA) for microsatellite data for the 4 clusters estimated with *STRUCTURE* (*K* = 4).

	<i>d.f.</i>	Sums of squares	Variance components	% of variation	<i>p</i> -value
<i>STRUCTURE</i> clustering = 4					
Among water bodies	3	79.1	0.23	13.56	0.001
Within water bodies	466	690.5	1.48	86.44	0.001
Total	469	769.6	1.71		

generation migrants, however, were assigned to other sampling sites within the study. Four individuals from A were assigned to C, both one individual from B was assigned to D and C respectively and three individuals from D were assigned to C.

Estimation of Selfing Rates

The inbreeding coefficient F_{IS} of the sampling sites ranged from 0.31 to 0.57 with an overall mean of 0.51. The selfing rate s estimated from F_{IS} ranged from 0.02 to 0.8, the overall mean was 0.34. The selfing rate derived with *RMES* (David et al., 2007) differed from the former estimate not so much in the mean, rather in the variance, as $s(g_2)$ ranged from 0 to 0.9 with an overall mean of 0.33. The Maximum Likelihood estimate of $s_{(ML)}$ ranged from 0.01 to 0.84 with an overall mean of 0.15.

DISCUSSION

Observed Local Population Structure Does Not Match Expectations from Active Dispersal

Estimating dispersal from observational data (Cameron & Williamson, 1977) or in combination with computer simulation has been successfully applied before in several studies on land snails (Baur & Baur, 1993; Pfenninger et al., 1996). These studies showed that simulated dispersal was similar to dispersal actually measured in natural habitats which justified its application in our study.

From active dispersal alone, we would have expected a homogeneous population within water body A and isolation by distance in water body C because: (i) the estimated neighbourhood where no differentiation may be expected comprises ~130 m of shoreline, which approximately corresponds to the shoreline of water body A, and (ii) our marker system would probably not be able to distinguish even a multiple of this measure. Nevertheless, sensitivity analysis of our data proved their suitability for correctly supporting the null hypothesis of no differentiation. However, the population differentiation inferred by nuclear and mitochondrial markers was much larger than expected (Table 4). There are several possible explanations for these discrepancies. First, the direct estimate might be exaggerated due to our study setup because (i) natural water bodies with diverse habitat structure might reduce the actual

neighbourhood size range due to preference of certain spots (Bovbjerg, 1968), and (ii) the density in the artificial pond might have been too high, which may have lead to crowding effects. However, since snails in most water bodies occurred in much higher abundances than in the study and were uniformly distributed along the shoreline (personal observation MP), these two issues should be of minimal importance. Second, the parameters from which neighbourhood size was calculated could have been derived from incorrect assumptions. For example, measurement of the temperature dependence of activity might not translate in a linear fashion to the field. Snails were kept in different temperature regimes for only one hour, so the metabolism might have lagged behind the actual temperature or the recorded distances might just show acclimatisation activity (Bailey & Johnston, 2005); for example, the attempt to escape high water temperatures that would have seized after a while. However, the linear response to temperature shifts argues against this latter possibility. Rather the relative correction of the dispersal distances measured in the field avoided the introduction of a systematic lag bias. Also a non-linear dependence of air temperatures (as reflected in the climate data) to water temperature would have biased the simulation results. However, except for water bodies influenced by geothermic or anthropologic activity, temperatures of surface water bodies, such as those observed here usually correspond in a linear fashion to air temperature.

The use of a *Radix balthica* population from outside the valley for the dispersal study might have biased the estimate if dispersal activity is population specific (Terblanche et al., 2009). Heritable variation in dispersal activity cannot be excluded; however, it is highly unlikely that differences on the order of magnitudes necessary to explain the discrepancy exist among the populations used for the direct approach and those observed in the field.

Third the mixed mating system could influence the population structure. High selfing rates, which ranged from 0 to 0.9 (mean 0.33; Table 1) in our study, should increase local inbreeding, thereby strongly decreasing the actual neighbourhood range (especially in the case of complete selfing) and thus lead to stronger spatial differentiation. However, in this case we would expect a pronounced isolation-by-distance pattern within water bodies at dispersal-drift equilibrium, which is not the case.

To explain the observed population structure based on neighbourhood range alone, the neighbourhood must not exceed a few square meters, which seems very unlikely, given the observational data. Even though we cannot be sure that we did not overestimate the neighbourhood range, lack of active dispersal is probably not sufficient to explain the structure observed. A non-equilibrium situation therefore remains as the most probable explanation.

Independent colonisation of the valley from several sources in combination with metapopulation dynamics and the mixed mating system may provide the processes causing the observed pattern. The haplotype and nuclear diversity found in the valley encompassed a substantial fraction of the diversity in the entire species range (Cordellier & Pfenninger, 2009), indicating colonisation of the valley from different sources. Indeed, the deviation of observed data from expectations results mainly from the presence of different nuclear clusters/mitochondrial lineages at certain sampling points within water bodies (Fig. 1). For example, sampling point A7 is differentiated from the other sampling points in water body A. This part is inhabited by a different lineage, indicating two independent colonisation events.

The weak albeit significant association of mitochondrial lineages with nuclear clusters indicated that there is either high ongoing gene-flow, constantly supplying individuals from the same populations or that the mixed mating system retards complete mixing of individuals from several immigration events in the past. The low estimate of first generation migrants both from within the valley and from the entire species range argues for the latter scenario.

Absence of the species from many apparently suitable water bodies during the sampling program, but subsequent colonisation of some (personal observation MP and TH) indicates a substantial metapopulation dynamic, which may influence population structure strongly. Metapopulation dynamics with frequent extinction and recolonisation events are common in other freshwater snails (Viard et al., 1997; Charbonnel et al., 2002; Facon & David, 2006). Since *Radix* shells are very thin-gauge, it is not possible to find empty shells which could give a hint at past occupations from other water bodies in the valley.

While the snails are not able to cross land barriers, passive dispersal must be assumed. For freshwater snails in particular, waterfowl have been suggested as dispersal vectors (Malone,

1965; Boag, 1986; Bilton et al., 2001). This is even more likely since land snails can survive passage through a bird's gut (Wada et al., 2011) and freshwater snails passage through a fish's gut (Brown, 2007). However, direct data on the qualitative and quantitative impact of bird-mediated dispersal is lacking and thus urgently needed to fully understand the population dynamics of freshwater invertebrates.

The fine-scale population structure here inferred by nuclear and mitochondrial markers thus reflects the species range-wide structure (Pfenninger et al., 2011), shaped by processes such as long-range dispersal, large-scale metapopulation dynamics and low levels of local passive dispersal. Active dispersal had, even in smallest water bodies, a nominal impact on local population structure. Furthermore, the hereby formed structure is preserved by the mixed mating system. We found no evidence that local processes to influence the observed structure.

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TH and MS performed the molecular work, analysed the data and drafted the manuscript, AP helped with the simulation models, MP designed the study, did the fieldwork, programmed the simulation models, analysed the data and contributed to writing the manuscript. All authors have approved the final version of the manuscript.

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